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Article

Impact of Carbon Fixation, Distribution and Storage on Terpene Production in Euryhaline Cyanobacteria

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Abstract: Terpenes are high-value chemicals which can be produced by engineered cyanobacteria from sustainable resources, solar energy, water and CO₂. We previously reported that the euryhaline unicellular cyanobacteria *Synechocystis* PCC 6803 (S.6803) and *Synechococcus* PCC 7002 (S.7002) produce farnesene and limonene, respectively, more efficiently than other terpenes. In the present study, we attempted at enhancing farnesene production in S.6803 and limonene production in S.7002. Practically, we tested the influence of key cyanobacterial enzymes acting in carbon fixation (RubisCO, PRK, CcmK3 and CcmK4), utilization (CrtE, CrtR and CruF) and storage (PhaA and PhaB) on terpene production in S.6803, and we compared some of the findings with the data obtained in S.7002. We report that the overproduction of RubisCO from S.7002 and PRK from *Cyanothece* PCC 7425 increased farnesene production in S.6803, but not limonene production in S.7002. The overexpression of the *crtE* genes (synthesis or terpene precursors) from S.6803 or S.7002 did not increased farnesene production in S.6803. In contrast, the overexpression of the *crtE* gene from S.6803, but not S.7002, increased farnesene production in S.7002, emphasizing the physiological difference between these two model cyanobacteria. Furthermore, the deletion of the *crtR* and *cruF* genes (carotenoid synthesis) and *phaAB* genes (carbon storage) did not increase the production of farnesene in S.6803. Finally, as a containment strategy of genetically modified (GMO) strains of S.6803 we report that the deletion of the *ccmK3K4* genes (carboxysome for CO₂ fixation) did not affect the production of limonene but decreased the production of farnesene in S.6803.

Keywords: *Synechocystis* PCC 6803; *Synechococcus* PCC 7002; farnesene; limonene; RubisCO; phosphoribulokinase; carboxysome; geranylgeranyl pyrophosphate synthase; β-carotene hydroxylase; γ-carotene hydroxylase

1. Introduction

Terpenes constitute a large family of chemicals produced by plants in low quantities and consequently at high prices. They are used for the production of fragrances, pharmaceuticals, pesticides, solvents and potentially biofuels [1,2]. All terpenes (general formula (C₅H₈)_n) derive from the five-carbons (C₅) building blocks dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). The head-to-tail covalent linkage of DMAPP and IPP catalyzed by the geranyl diphosphate synthase (GPPS) enzyme, generates the geranyl diphosphate (GPP) precursor of monoterpenes (C₁₀H₁₆), such as limonene. Then, the addition of another IPP unit on GPP catalyzed by the farnesyl diphosphate synthase (FPPS), forms the farnesyl pyrophosphate (FPP) precursor of sesquiterpenes (C₁₅H₂₄), such as farnesene (Figure 1).

Besides plants, which are better used for food, cyanobacteria, the robust photosynthetic prokaryotes colonizing our planet, can be used for the ecologically responsible production of terpenes

from sunlight and CO₂ (for reviews see [1,2]). Cyanobacteria fix CO₂ using the Calvin-Benson-Bassham (CBB) pathway in which the key enzyme ribulose biphosphate carboxylase/oxygenase (RubisCO) catalyzes the addition of one molecule of CO₂ to one molecule of ribulose biphosphate produced by the other key enzyme phosphoribulokinase (PRK). In parallel, they have developed the carboxysome sub-cellular compartment (assembled from various shell proteins such as CcmK3 and CcmK4) to encapsulate RubisCO in a CO₂-rich environment that favor its carbon-fixing (carboxylase) activity over its detrimental oxygenase activity (for reviews see [3,4]). A part of the photosynthetically-fixed carbon is used by the methylerythritol-4-phosphate (MEP) pathway to produce DMAPP and IPP (Figure 1), which are transformed by a geranylgeranyl pyrophosphate synthase (CrtE) into GPP, FPP, and GGPP which is used by the carotene hydroxylases (CrtR and CruF) to produce chlorophyll and carotenoids [1,2,5]. GPP and FPP can also be transformed into terpenes, following introduction and expression in cyanobacteria of synthetic terpene-synthase encoding genes adapted to the cyanobacterial codon usage [1,2]. The unicellular euryhaline cyanobacteria *Synechocystis* PCC 6803 (hereafter S.6803) and *Synechococcus* PCC 7002 (S.7002, costal organism) are interesting for this purpose. They have a good genetics and they grow well in sea and brackish waters to preserve freshwater resources for agriculture [6].

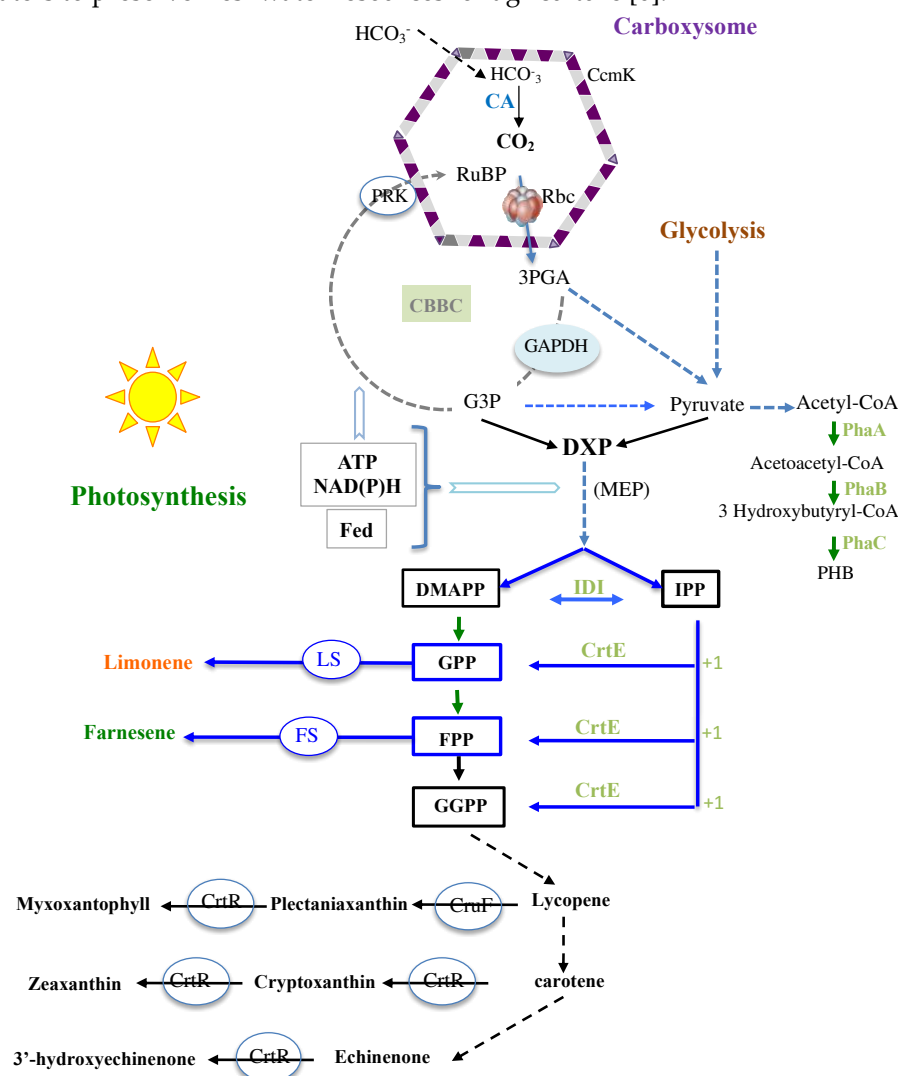


Figure 1. Schematic representation of the metabolic pathway and key compounds involved in the synthesis of terpenes from CO₂. Abbreviations. CA: carbonic anhydrase; CBB: Calvin Benson Bassham cycle (consuming 1 NADPH, 1 ATP); CrtE: geranylgeranyl pyrophosphate synthase; DMAPP: dimethylallyl pyrophosphate; DXP: 1-deoxy-D-xylulose-5-phosphate; Fed: ferredoxin; FPP: farnesyl pyrophosphate; FS: farnesene synthase; GAPDH: glyceraldehyde-3P-dehydrogenase; GPP: geranyl pyrophosphate; GPP: geranyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; G3P:

glyceraldehyde-3-phosphate; 3PGA: 3-phosphoglycerate; IPP: isopentenyl pyrophosphate; IDI: isopentenyl-diphosphate isomerase; LS: limonene synthase; MEP: methylerythritol 4-phosphate (it consumes 2 NADPH, 1 ATP & 1 CTP); phaA: acetyl-CoA acetyltransferase; phaB: Acetoacetyl-CoA reductase; phaC: PHB synthase; PRK: phosphoribulokinase; Rbc: ribulose biphosphate carboxylase; RuBP: ribulose-1,5-bisphosphate.

In the frame of our exploration of the ability of physiologically-different cyanobacteria for the photoproduction of chemically-different terpenes, we previously engineered several cyanobacteria by cloning codon-adapted version of terpene synthase-encoding genes, expressed from the strong lambda-phage pR-promoter, in a RSF1010-derived replicative plasmid or in a neutral chromosome site [7–10]. The results showed that S.6803 produces farnesene better than bisabolene, limonene, santalene and pinene [8], whereas S.7002 produces limonene more efficiently than bisabolene and pinene [10]. In the present study we used several genetic strategies to address the improvement of farnesene production in S.6803 and limonene production in S.7002.

2. Results and Discussion

2.1. The Overexpression of the RubisCO genes from *Synechococcus* PCC 7002 Increases Farnesene Production in *Synechocystis* PCC 6803

Previous studies showed that overexpressing the RubisCO-encoding genes can improve cell growth and/or the photosynthetic production of high value chemicals in S.6803 and/or S.7002 [11–14]. In this study, we attempted to improve farnesene production in S.6803 by introducing an extra-copy of the RubisCO-encoding genes, in the S.6803-ChrFS expressing the *Picea abies* α -farnesene synthase gene from a neutral site of its chromosome [8]. To increase the odd of improving RubisCO activity in S.6803, we used extra RubisCO genes from not only S.6803, but also physiologically-diverse cyanobacteria studied in our laboratory. These are namely: *Cyanothece* PCC 7425 (hereafter C.7425) and S.7002 where their RubisCO genes constitute the *rbcLXS* operon encoding the large (RbcL) and small (RbcS) RubisCO subunits, and the RbcX chaperon (See Cyanobase). We also used the *rbcLS* operon of *Synechococcus elongatus* PCC 7942 (S.7942) where *rbcX* is located away from *rbcLS* [15,16].

These RubisCO operons were PCR amplified from their respective cyanobacterial genomes (Figure S1), using oligonucleotides (Table S1) that introduced convenient restriction site upstream of the ATG start codon of *rbcL* (*NdeI* for S.6803, S.7002 and S.7942, and *XhoI* for C.7425) and downstream of the TAA stop codon of *rbcS* (*EcoRI* for S.6803, *PvuII* for both S.7002 and S.7942, and *BspEI* for C.7425). After restriction, the RubisCO operons were cloned downstream of the strong pR promoter [17] of the RSF1010-derived replicative pC plasmid vector [18] opened with the same enzymes. The resulting Sm^R/Sp^R plasmids, pCrbc6803, pCrbc7002, pCrbc7942 and pCrb7425 (Figure S1 and Table S2) and the (empty) pC control vector, were introduced by conjugation in the S.6803-ChrFS engineered strain that produces farnesene [8]. In each case, two independent Sm^R/Sp^R clones were selected and analyzed by PCR and DNA sequencing (Figure S1). The data showed that all these pC-derived plasmids, pCrbC7425, pCrbC7942, pCrbC6803 and pCrbC7002, replicate stably in S.6803 with no impact on cell growth, like the pC control vector (Figure 2). The resulting recombinant strains, which also have their indigenous chromosomal RubisCO genes, are represented in Figure S2.

The production of farnesene by the S.6803-chrFS strains propagating the RubisCO-producing plasmids pCrbc6803, pCrbc7002, pCrbc7425 and pCrbc7942 was measured over 21-days periods of photoautotrophic growths, as previously described [8]. GC-MS analysis of the terpene-trapping dodecane overlay samples from all tested strains showed a peak with similar retention time and ion chromatogram than a pure standard of α -farnesene. As compared to the S.6803-chrFS control strain, the level of farnesene production driven by the pCrbc6803, pCrbc7002, pCrbc7425 and pCrbc7942 plasmids showed that high-level expression of the RubisCO genes from S.7002, but not C.7425, S.6803 and S.7942, enhanced farnesene production (about 2 folds at day 21, Figure 2). This finding suggests that the RubisCO of S.7002 is more active in S.6803 than the other tested RubisCO, including the endogenous enzyme of S.6803.

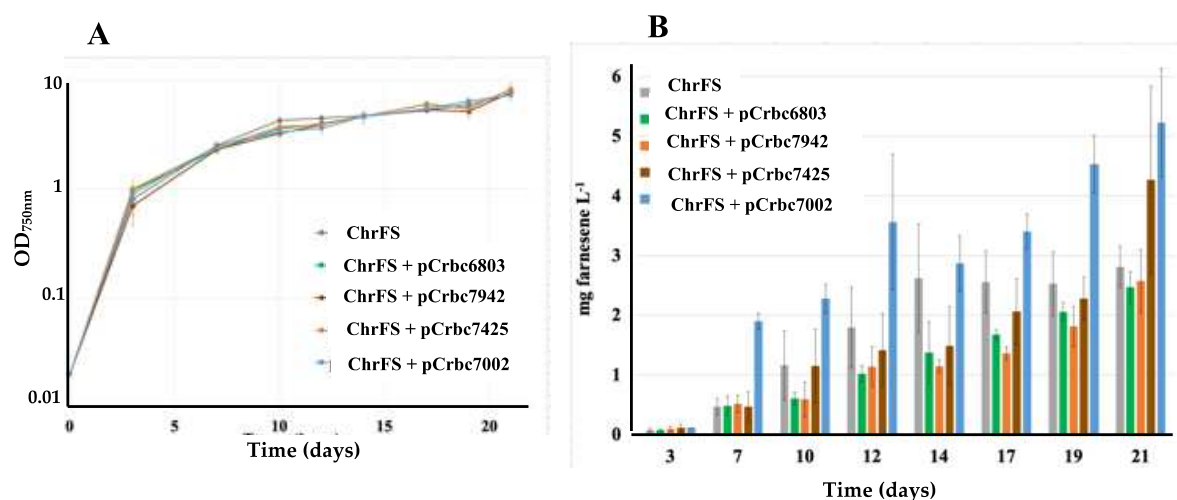


Figure 2. Simultaneous analysis of growth (A) and farnesene production (B) of the *Synechocystis* PCC 6803 strains harboring the strongly-expressed genes encoding farnesene-synthase (FS) in a neutral chromosomal site (chrFS) and the RubisCO (*rbc*) genes, in a pC-derived replicative plasmid (pCrbc). These plasmids encode the RubisCO enzymes from the distantly-related unicellular cyanobacteria *Cyanothece* PCC 7425 (pCrbc7425), *Synechococcus* PCC 7002 (pCrbc7002), *Synechococcus* PCC 7942 (pCrbc7942) and *Synechocystis* PCC 6803 (pCrbc6803). Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 21 days. Error bars represent standard deviation from biological triplicates.

2.2. The Overexpression of the Phosphoribulokinase Gene from *Cyanothece* PCC 7425 Increases Farnesene Production in *Synechocystis* PCC 6803

As the elimination of the CP12 negative regulator of the phosphoribulokinase (PRK) enzyme was shown to improve terpene production in S.6803 [9], we decided to overexpress the *prk* gene as an attempt to enhance farnesene production. To increase the odd of enhancing PRK activity, we decided to clone an extra copy of *prk* from not only S.6803, but also C.7425, S.7002 and S.7942, in the pC plasmid vector. The *prk* genes were PCR amplified from their respective cyanobacterial genome, using oligonucleotide primers (Table S1) that embedded their ATG start codon in a *Nde*I restriction site (CATATG), and introduced an *Eco*RI site downstream of their TAA (S.6803) or TAG (S.7002, S.7942 and C.7425) stop codons. After restriction with both *Nde*I and *Eco*RI, these genes were cloned in pC opened with the same enzymes, and transformed to *E. coli*. All attempts to clone the *prk* genes from S.6803, S.7002 and S.7942 in pC were unsuccessful, indicating that cyanobacterial PRKs are toxic to *E. coli*. Supporting this interpretation, previous cloning in *E. coli* of the *prk* gene from S.7942 [19] and S.6803 [20], for *in vitro* analysis of PRK, employed an inducible promoter system that decoupled *E. coli* growth from the production of PRK.

However, we were able to clone the *prk* gene from C.7425 downstream of the strong constitutive pR promoter of the pC plasmid (Table S2), suggesting that PRK₇₄₂₅ is somehow less active in *E. coli* than the other cyanobacterial PRKs presently tested. The resulting plasmid, pCprk7425 (Table S2), was introduced by conjugation in the S.6803-chrFS strain expressing the farnesene synthase gene from its chromosome [8]. A Sm^R/Sp^R clone (Figure S2) was analyzed to show that pCprk7425 does not alter the growth of S.6803 (Figure 3). The production of farnesene during the photoautotrophic growth of the S.6803-chrFS-pCprk7425 reporter strain (Figure S2) was analyzed for 21-days, and compared to that of the S.6803-chrFS control strain. The data showed that increasing the expression of *prk* enhances (about three-folds) the production of farnesene in S.6803 (Figure 3). This result is consistent with our previous report on CP12 [9] and the above finding that overexpressing RubisCO genes improved farnesene production in S.6803 (Figure 2). Collectively, these data indicate that increasing CO₂ fixation enhances terpene production in S.6803.

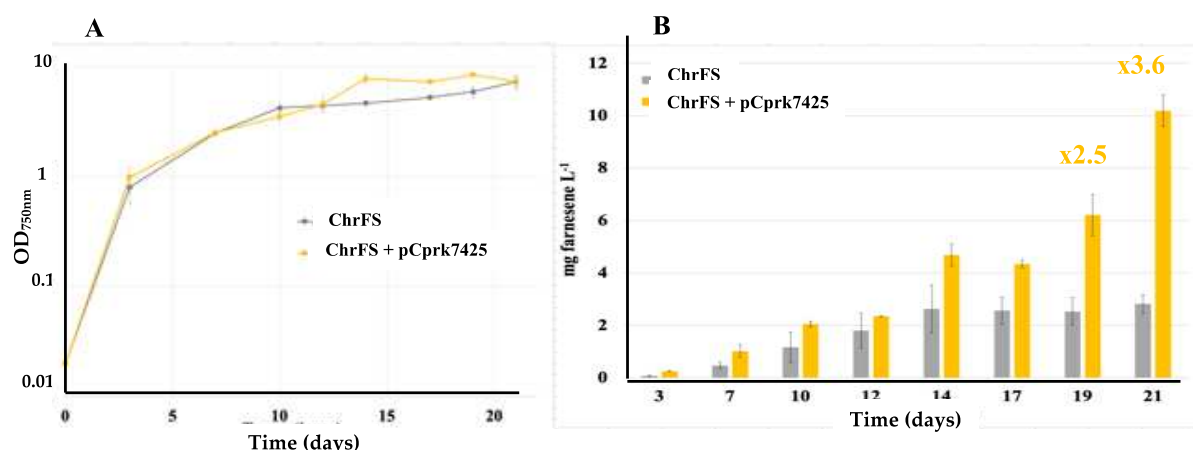


Figure 3. Simultaneous analysis of growth (A) and farnesene production (B) of the *Synechocystis* PCC 6803 strain harboring the strongly expressed genes encoding farnesene synthase (FS) and phosphoribulokinase, in a neutral chromosomal site (chrFS) and a pC-derived replicative plasmid, respectively. This pCprk7425 plasmid encodes the PRK enzyme from *Cyanothece* PCC 7425, a unicellular cyanobacterium distantly related to both *Synechocystis* PCC 6803 and *Synechococcus* PCC 7002. Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 21 days. Error bars represent standard deviation from biological triplicates.

2.3. The Overexpression of the Genes Encoding Rubisco and PRK Enzymes does not Increase Limonene Production in *Synechococcus* PCC 7002

We also attempted to improve limonene production in the previously described S.7002 strain, named S.7002-chrLS, which harbors the *Mentha spicata* 4S-limonene synthase gene in a neutral chromosome site [10]. Therefore, the pC-derived plasmids overexpressing the studied phosphoribulokinase gene (pCprk7425) or RubisCO operons (pCrbc6803, pCrbc7002, pCrbc7425 and pCrbc7942) were introduced by conjugation in S.7002-chrLS. The production of limonene of the resulting reporter strains (Figure S3) appeared to be similar to that of the S.7002-chrLS control strain (Figure 4). Collectively, the present findings show that the standard photoautotrophic growth of both S.6803 and S.7002 is not limited by the natural abundance of their indigenous RubisCO and PRK enzymes. Nevertheless, the production of terpene is limited by the natural abundance of the RubisCO and PRK in S.6803, not in S.7002. This difference reminds us that because a model cyanobacterium is essentially a model of itself, it is therefore important to study several cyanobacteria in parallel (in the same laboratory) to better understand their common and specific properties, and exploit this knowledge for biotechnological purposes.

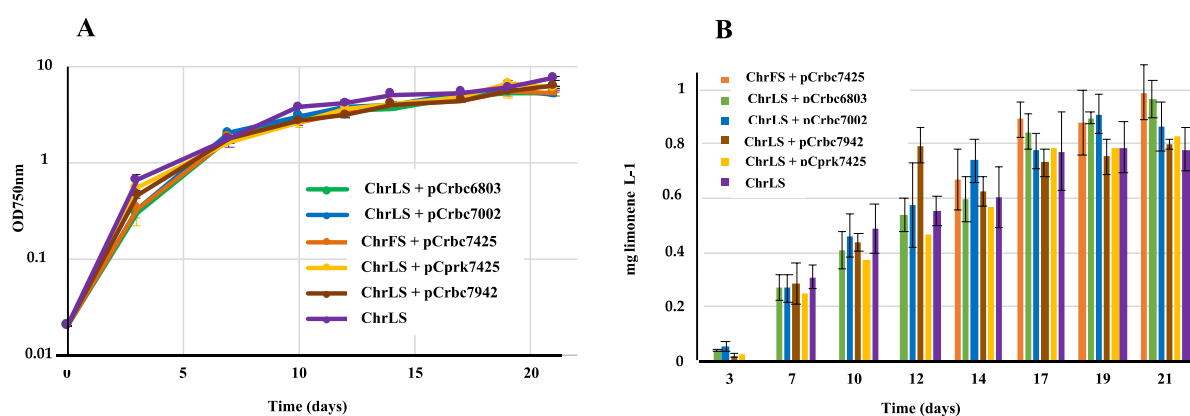


Figure 4. Simultaneous analysis of growth (A) and limonene production (B) of the *Synechococcus* PCC 7002 strains harboring the strongly-expressed limonene-synthase gene (LS) in a neutral chromosomal

site (chrLS) and a pC-derived plasmid expressing either the phosphoribulokinase gene from *Cyanothece* PCC 7425 (pCprk7425) or the RubisCO genes from *Cyanothece* PCC 7425 (pCrbc7425), *Synechococcus* PCC 7002 (pCrbc7002), *Synechococcus* PCC 7942 (pCrbc7942) or *Synechocystis* PCC 6803 (pCrbc6803). Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 21 days. Error bars represent standard deviation from biological triplicates.

2.4. The Overexpression of the *CrtE* Genes from *Synechocystis* PCC 6803 or *Synechococcus* PCC 7002 Decreases the Production of Both Farnesene and Limonene in *Synechocystis* PCC 6803

One of the challenges in using cyanobacteria for terpene production is the competition for prenyl pyrophosphates (GPP, FPP and GGPP) between the synthesis of photosynthetic pigments and the intended terpene (Figure 1). As most cyanobacteria, such as S.6803 and S.7002, have a single GGPP synthase (named *CrtE*) for the sequential synthesis of GPP, FPP and GGPP [5,21–23], several authors overexpressed synthetic genes encoding FPPS (*IspA*) from *E. coli* [24,25] or GPPS from plants [26–28] to improve (2–3 folds) terpene production in S.6803 or S.7942 (S.7002 was not tested). These moderate improvements could result from these heterologous enzymes having a low activity and/or stability in cyanobacteria, an eventuality pleading in favor of testing cyanobacterial *CrtE* enzymes. In this context, the structural differences in the *CrtE* enzymes from S.7002 and S.6803 [5,23] prompted us to overexpress the *crtE* genes from S.6803 (hereafter *crtE*_{S.6803}) and S.7002 (*crtE*_{S.7002}) to attempt at enhancing terpene production in both S.6803 and S.7002. Therefore, the protein coding sequences of the *crtE*_{S.6803} and *crtE*_{S.7002} genes were PCR amplified from the genomes of S.6803 or S.7002, using specific oligonucleotides (Table S1) flanking them with a unique restriction site upstream of their start codon, and another unique restriction site behind their stop codon (Table S2). After restriction, these DNA fragments were cloned downstream the *Picea abies* α -farnesene synthase gene (FS) or *Mentha spicata* 4S-limonene synthase gene (LS) of our previously described pCFS and pCLS plasmids [7,8] opened with the same enzymes (*Xho*I and *Eco*RI for pCFS, and *Eco*RI and *Acl*I for pCLS). The resulting plasmids (Table S2) pCFS*crtE*_{S.6803} and pCFS*crtE*_{S.7002} (expression of the FS*crtE*_{S.6803} or FS*crtE*_{S.7002} operons), and pCLS*crtE*_{S.6803} and pCLS*crtE*_{S.7002} (expression of the LS*crtE*_{S.6803} or LS*crtE*_{S.7002} operons) were introduced by conjugation in S.6803 (Figure S4) where they appeared to replicate stably (Figure S5). The resulting S.6803 reporter strains (Figure S4), which also expressed their indigenous *crtE* gene from their chromosome, grew similarly to the control strains propagating the pC, pCFS or pCLS control plasmids (Figure 5). The production of farnesene directed by the pCFS*crtE*_{S.6803} and pCFS*crtE*_{S.7002} plasmids was lower (about three-folds) than the level driven by the pCFS control plasmid (Figure 5). Similarly, the production of limonene directed by the pCLS*crtE*_{S.6803} and pCLS*crtE*_{S.7002} plasmids were lower (about six-folds and three-folds, respectively) than the level driven by the pCLS control plasmid (Figure 5). Collectively, these data indicate that the natural abundance of the *CrtE* enzyme is not limiting terpene production in S.6803. The present finding that an increased level of *CrtE* actually decreases terpene production suggests that the additional molecules of the GGPP metabolite synthesized by the extra molecules of *CrtE* might inhibit the MEP pathway and/or the studied terpene synthases, similarly to the feedback inhibitions caused by the accumulation of IPP [2]. In addition, the increased level of *CrtE* might negatively influence the hypothetical metabolic pairing of *CrtE* with the GGPP phosphatase [23], which could somehow participate to terpene synthesis.

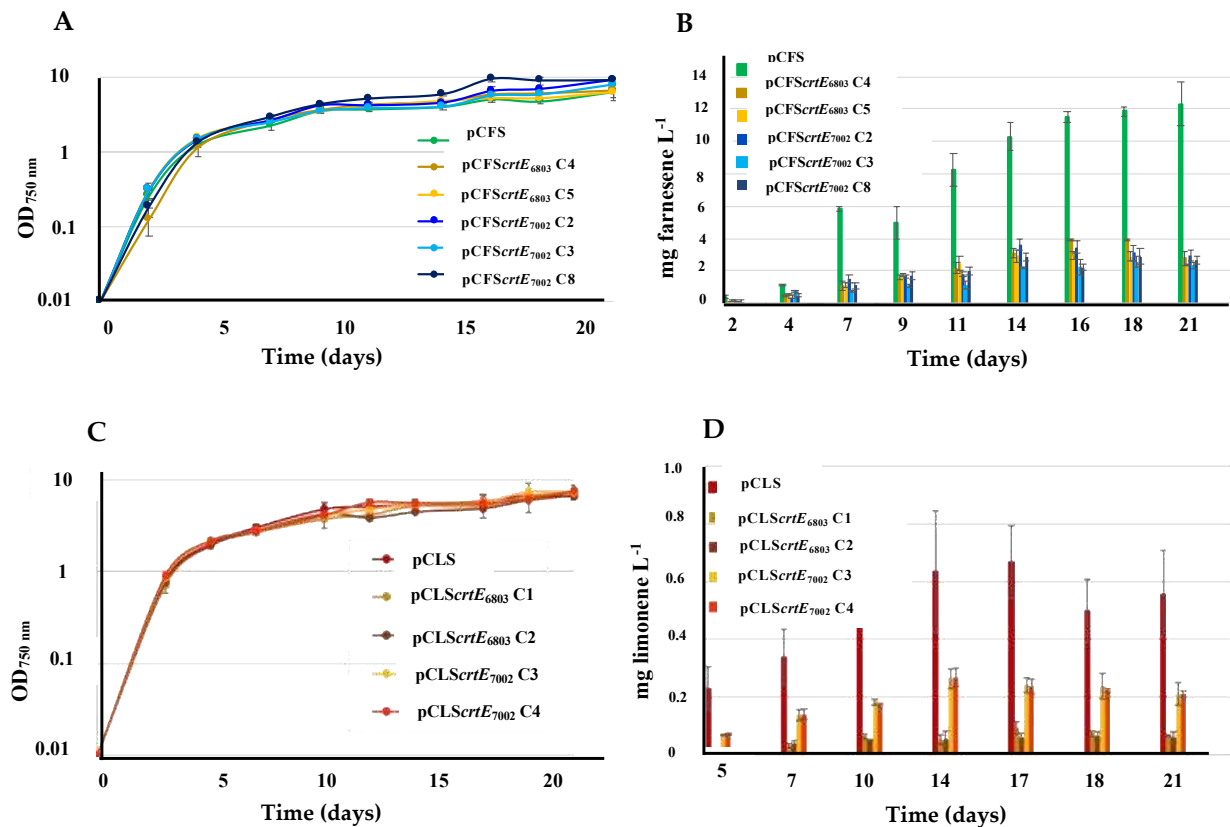


Figure 5. Simultaneous analysis of growth (A,C) and terpene production (B,D) of *Synechocystis* PCC 6803 strains harboring a pC-derived plasmid strongly expressing either the farnesene synthase gene (pCFS; A,B) alone or transcriptionally fused to the *crtE* gene from either *Synechocystis* PCC 6803 (pCFSrtE₆₈₀₃) or *Synechococcus* PCC 7002 (pCFSrtE₇₀₀₂), or the limonene synthase gene (pCLS; C,D) alone or transcriptionally fused to a *crtE* gene (pCLSrtE₆₈₀₃ or pCLSrtE₇₀₀₂). Several clones were studied, as indicated by C1-C8. Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 21 days. Error bars represent standard deviation from biological triplicates.

2.5. The Overexpression of the *CrtE* Gene from *Synechocystis* PCC 6803, but not *Synechococcus* PCC 7002 Increases Farnesene Production in *Synechococcus* PCC 7002

The expression of extra copy of the *crtE* gene on terpene production in S.7002 was tested as follows. The pCFSrtE₆₈₀₃ and pCFSrtE₇₀₀₂ plasmids overexpressing the operonic genes encoding the *Picea abies* α -farnesene synthase and the *CrtE* enzyme from either S.6803 or S.7002, were introduced by conjugation in S.7002, along with the pCFS control plasmid expressing only the farnesene synthase gene (Figure S6). All plasmids appeared to replicate stably (Figure S7) without affecting cells growth (Figure 5). As compared to pCFS, the pCFSrtE₆₈₀₃ and pCFSrtE₇₀₀₂ reporter plasmids increased and (slightly) decreased farnesene production in S.7002, respectively (Figure 6). These data suggest that the activity of the *CrtE*₆₈₀₃ and *CrtE*₇₀₀₂ enzymes might be different in S.7002.

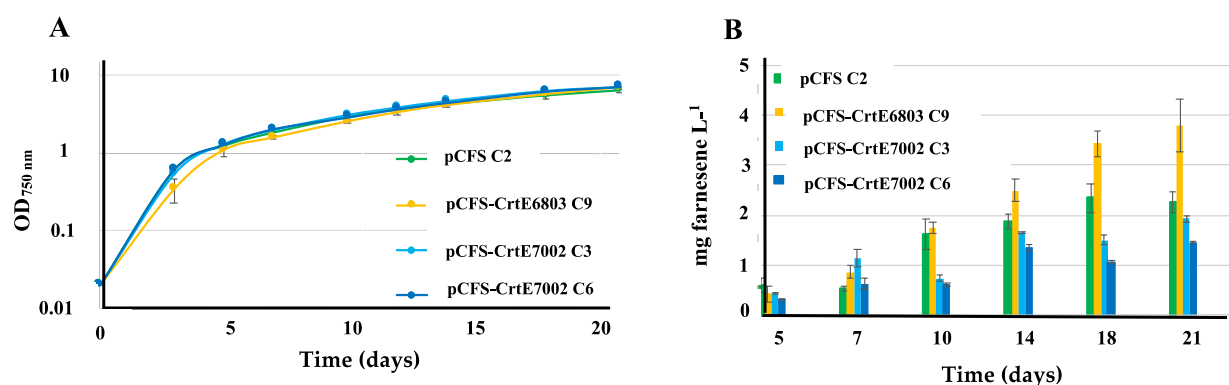


Figure 6. Simultaneous analysis of growth (A) and farnesene production (B) of *Synechococcus* PCC 7002 strains harboring a pC-derived plasmid strongly expressing either the farnesene synthase gene (pCFS) alone or transcriptionally fused to the *crtE* gene from either *Synechocystis* PCC 6803 (pCFS-*CrtE*₆₈₀₃) or *Synechococcus* PCC 7002 (pCFS-*CrtE*₇₀₀₂). Three clones were studied, as indicated by C3, C6 and C9. Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 21 days. Error bars represent standard deviation from biological triplicates.

2.6. The Deletion of PHB Synthesis Genes does not Increase the Photoproduction of Terpenes in *Synechocystis* PCC 6803

In S.6803, most of the photosynthetically-fixed carbon is used for the production of biomass and abundant carbon stores such as glycogen and polyhydroxybutyrates (PHB) [29,30], while only 5% of the carbon is allocated to the MEP pathway for the synthesis of photosynthetic pigments (carotenoids, chlorophyll and quinone) [1,31]. As an attempt to redirect the carbon flux toward the MEP pathway to enhance terpene production in S.6803, we deleted the two adjacent genes *phaAB* encoding the acetoacetyl-CoA thiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) enzymes, which catalyze the first step in PHB synthesis [32–34]. Therefore, a Δ *phaAB*::Km^R DNA cassette (Table S2) harboring a transcription-terminator less Km^R marker in place of most of the *phaA* and *phaB* collinear genes (from codon 93 of *phaA* to codon 141 of *phaB*) was constructed, as follows (Figure S8). The two 300 bp chromosomal DNA regions flanking the *phaA-phaB* DNA region to be deleted were synthesized by TWIST Bioscience as a single DNA segment harboring an *EcoRV* restriction site in its middle where we cloned the Km^R marker (a *HincII* segment from pUC4K) in the same orientation as the *phaAB* genes that it replaced (Figure S8). This Δ *phaAB*::Km^R DNA cassette was then transformed to S.6803, where DNA recombination occurring in the homologous DNA platforms flanking the Km^R gene integrated this marker in place of the *phaAB* gene, in all copies of the S.6803 chromosome (Figure S8). Then, the Sm^R/Sp^R pC-derived plasmids pCFS and pCLS [7,8] were introduced by conjugation in the Δ *phaAB*::Km^R mutant (Figure S8 and (Figure S9)). In each case, several Sm^R/Sp^R/Km^R clones were studied. The data showed that the deletion of the PHB synthesis genes *phaAB* increase the photoproduction of neither farnesene nor limonene in S.6803 (Figure 7).

This approach of deleting PHB synthesis genes to redirect photosynthetically-fixed carbons toward terpene production could not be tested in S.7002 because it does not synthesize PHB [35].

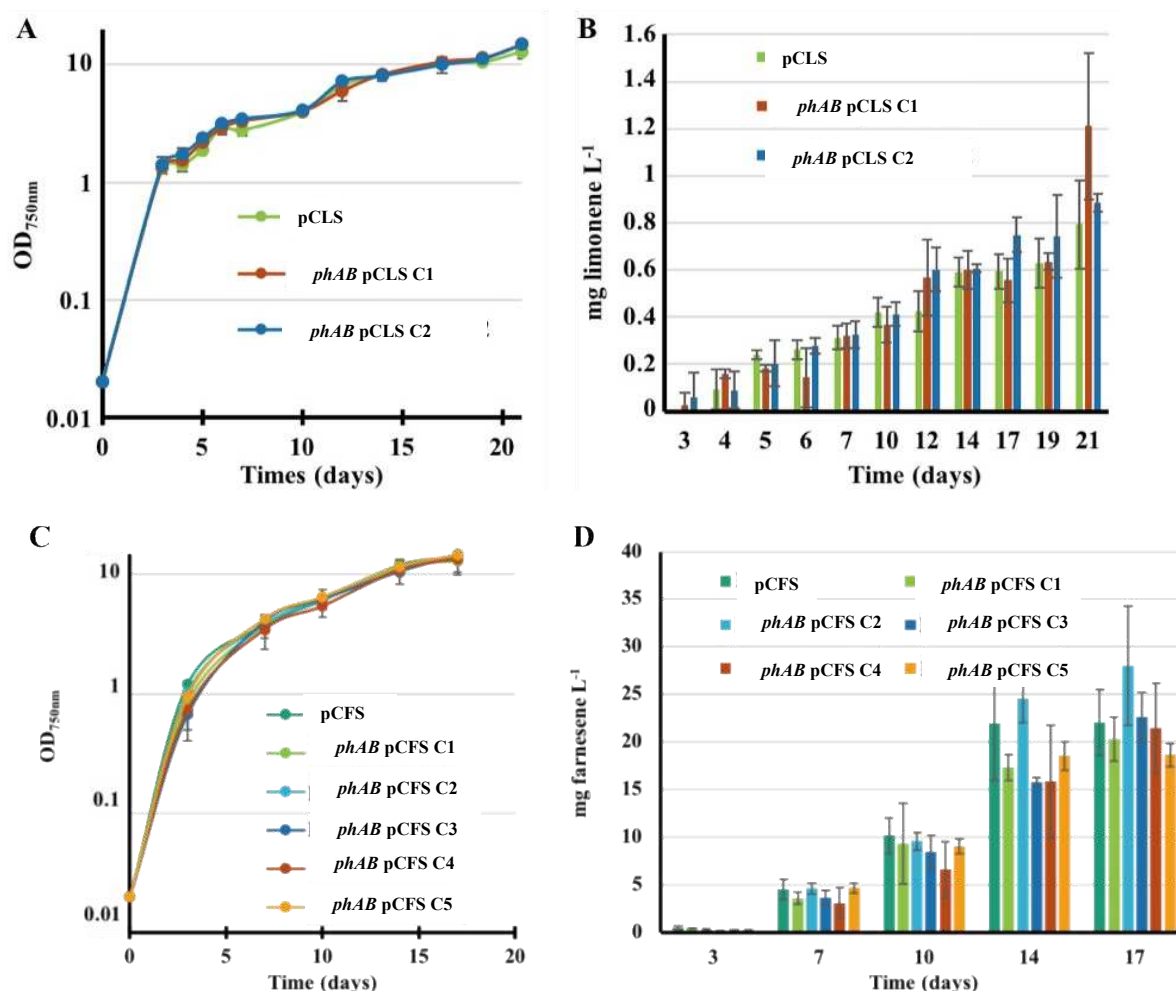


Figure 7. Simultaneous analysis of growth (A,C) and terpene production (B,D) of *Synechocystis* PCC 6803 strains WT or *DphaAB* mutant harboring a pC-derived plasmid strongly expressing either the limonene synthase gene (pCLS; A,B) or the farnesene synthase gene (pCFS; C,D). Several clones were studied, as indicated by C1-C5. Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 17 days. Error bars represent standard deviation from biological triplicates.

2.7. The Deletion of the Carotenoid Synthesis Genes *crtR* and *cruF* Decreases the Production of Farnesene in *Synechocystis* PCC6803

Production of sesquiterpenes, such as farnesene, competes with the synthesis of photosynthetic pigments (chlorophyll and carotenoids) because they use the same precursor metabolite FPP [36] (Figure 1). To save FPP for a better farnesene production, we decided to decrease the consumption of the FPP-derived metabolite GGPP used for carotenoids synthesis (Figure 1). Therefore, we deleted the genes *crtR* (β -carotene hydroxylase) and *cruF* (γ -carotene hydroxylase) acting in GGPP-consuming synthesis of carotenoids. For *crtR* deletion, a *DcrtR::Km^R* DNA cassette (Table S2) was constructed by replacing the first 82 codons of *crtR* by a transcription-terminator-less *Km^R* marker, as follows. The two 300 bp chromosomal DNA regions flanking the first 82 codons of *crtR* to be deleted were synthesized by TWIST Bioscience as a single DNA segment harboring an *EcoRV* restriction site in its middle, where we cloned the transcription-terminator-less *Km^R* marker (a *HincII* segment from the pUC4K plasmid) in the same orientation as *crtR* (Figure S10). The *DcrtR::Km^R* DNA cassette was transformed to S.6803, where homologous DNA recombination replaced *crtR* by the *Km^R* marker, in all copies of the S.6803 chromosome (Figure S10). The resulting *DcrtR::Km^R* mutant grew as fit as the

WT strain under standard light, showing that *crtR* is not essential for the photoautotrophic growth of S.6803 as previously observed [37,38].

Then, the Sm^R/Sp^R pCFS plasmid was introduced by conjugation in the *DcrtR*::Km^R mutant (Figure S10), and the production of farnesene by two Sm^R/Sp^R/Km^R clones was analyzed during photoautotrophic growth (Figure 8). The data showed that the deletion of *crtR* does not increase the photoproduction of farnesene in S.6803 (it was decreased), unlike what was expected.

For *cruF* deletion, a *DcruF*::Km^R DNA cassette (Table S2) was constructed (Figure S12) by replacing an internal part of the *cruF* coding sequence (CS, from codon 100 to codon 271) by the same Km^R marker. The two 300 bp DNA regions flanking the 171 codons of *cruF* to be deleted were synthesized by TWIST Bioscience as a single DNA segment harboring an *EcoRV* site in its middle, where we cloned the Km^R marker in the same orientation as *cruF*. The *DcruF*::Km^R DNA cassette was verified by PCR and nucleotide sequencing, and then transformed to S.6803 where homologous DNA recombination replaced the 171 codons of *cruF* by the Km^R marker, in all copies of the S.6803 chromosome (Figure S12). The resulting *DcruF*::Km^R mutant grew as fit as the WT strain under standard light showing, for the first time, that *cruF* is not crucial for the photoautotrophic growth of S.6803.

Then, the Sm^R/Sp^R pCFS plasmid was introduced by conjugation in the *DcruF*::Km^R mutant (Figures S11 and S12), and the farnesene production of two independent Sm^R/Sp^R/Km^R clones was analyzed (Figure 8). As reported above in the case of *crtR*, the deletion of *cruF* did not increase, but decreased, the photoproduction of farnesene in S.6803 (Figure 8).

Since the strategy of deleting the *crtR* and *cruF* genes did not increase the production of farnesene in S.6803 it was not tested in S.7002.

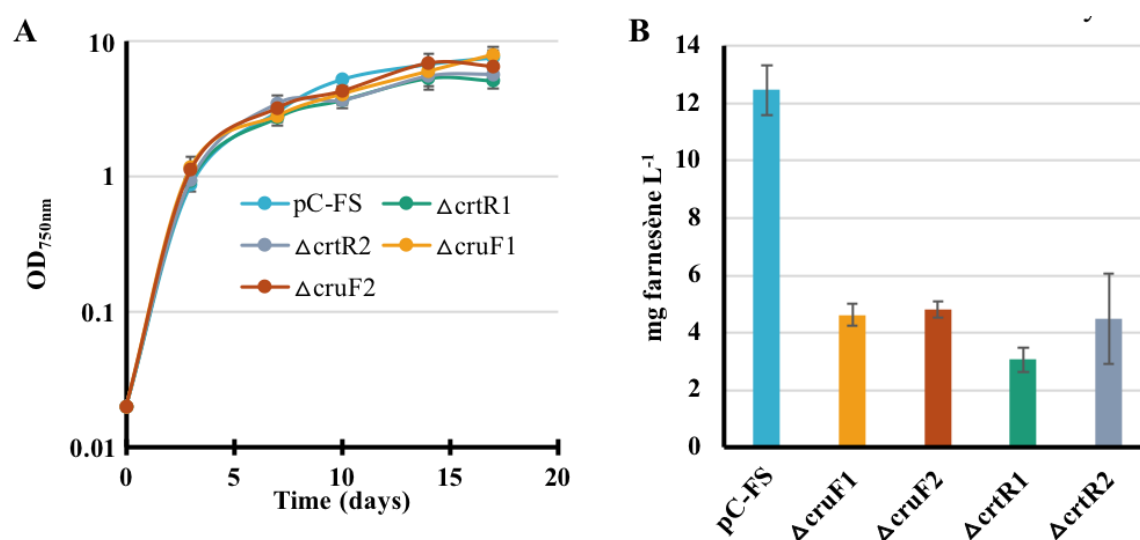


Figure 8. Simultaneous analysis of growth (A) and farnesene production (B) of *Synechocystis* PCC 6803 WT strain and two clones (1 and 2) of each of the *DcrtR* and *DcruF* mutants harboring the farnesene synthase production pCFS plasmid. Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay farnesene production (measured at day 10). Error bars represent standard deviation from biological triplicates.

2.8. Deletion of Both the *ccmK3* and *ccmK4* Genes Encoding Carboxysome Shell Proteins does not Alter the Production of Limonene but Decreases the Production of Farnesene in *Synechocystis* PCC 6803

Besides the engineering of genetically-modified cyanobacterial organisms (GMO) for the photosynthetic production of high-value chemicals, it is important to consider strategies to limit accidental release of these GMO in natural environments. For this purpose, an interesting target is the carbon concentrating mechanism (CCM) that cyanobacteria use to grow in the low CO₂ concentration of their natural aquatic biotopes. The CCM system uses the carboxysome sub-cellular compartment, assembled from various Ccm shell proteins, which encapsulates the RubisCO enzyme

in a CO₂-rich environment favoring its carbon-fixing (carboxylase) activity over its detrimental oxygenase activity (for reviews see [3,4]). As a biocontainment strategy, previous workers have deleted carboxysome genes of S.7002 and S.7942 GMO to impose a high-CO₂ requirement phenotype (HCR) preventing them from escaping from their cultivation photobioreactors [39,40]. Such a HCR phenotype did not negatively impact L-lactate production in S.7002 [39], but it decreased (about two fold) farnesene production in S.7942 [40]. Since the HCR containment of GMO has not yet been tested in S.6803, we decided to delete the *ccmK3* and *ccmK4* adjacent genes encoding the CcmK3 and CcmK4 carboxysome shell proteins which assemble as CcmK3-K4 hetero-hexamers in both S.6803 [41] and S.7942 [42]. These proteins were shown to be required for cell growth at low CO₂ in S.7942 [42,43], and for optimal photoautotrophic growth in S.6803 [44].

A *DccmK3K4::Km^R* DNA cassette (Table S2) was constructed to delete both the *ccmK3* and *ccmK4* colinear genes (from the start of *ccmK3* coding sequence (CS) to the stop codon of *ccmK4*). The 250 bp regions upstream the *ccmK3* CS and downstream the *ccmK4* CS were synthesized by TWIST Bioscience as a single DNA segment harboring a *SwaI* and a *BamHI* restriction sites where we cloned the Km^R marker (using the same enzymes) in the same orientation as the *ccmK3* and *ccmK4* CS it replaced (Figure S13). The *DccmK3K4::Km^R* grew as healthy as the WT strain in the Na₂CO₃-rich MM medium, but was unable to grow under atmospheric (low) CO₂ level (MM₀ medium), in agreement with some previous findings in S.7942 [42,43] and S.6803 [44].

Then, the Sm^R/Sp^R plasmids, pCFS and pCLS were introduced by conjugation in the *DccmK3K4::Km^R* double mutant, the production of terpenes in the resulting reporter strains (Figure S13) was analyzed during photoautotrophic growth in the Na₂CO₃-rich MM medium. The data showed that the double deletion of *ccmK3K4* did not alter the photoproduction of limonene (monoterpene, C₁₀) in S.6803 (Figure 9), as expected. In contrast, the deletion of *ccmK3K4* decreased the higher production of farnesene (sesquiterpene, C₁₅). Interestingly, these data showed that the more carbon atoms is used to produce the observed quantity of the studied terpene (the production of farnesene (C₁₅) is more important than that of limonene (C₁₀)) the more the production is decreased by the *DccmK3K4::Km^R* deletion.

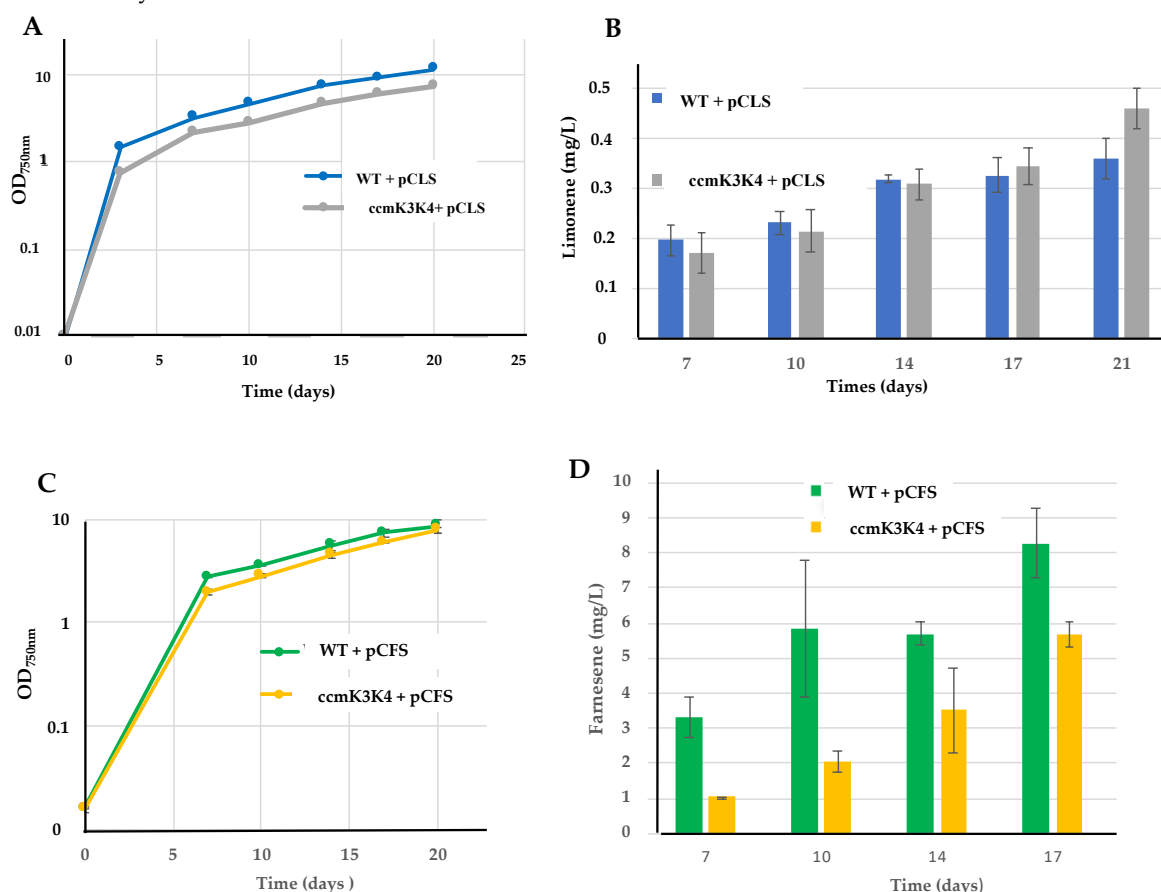


Figure 9. Simultaneous analysis of growth (A,C) and terpene production (B,D) of *Synechocystis* PCC 6803 strains WT or *DccmK3K4* mutant harboring a pC-derived plasmid strongly expressing either the limonene synthase gene (pCLS; A,B) or the farnesene synthase gene (pCFS; C,D). Cells were grown under standard photoautotrophic conditions in the presence of a dodecane overlay (20% vol/vol) to assay terpene production for 17 or 21 days. Error bars represent standard deviation from biological triplicates.

3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

E. coli strains used for DNA manipulations (TOP10 and NEB10 beta, Table S1) or conjugative transfer to S.6803 and S.7002 (CM404, [17]) of pC-derived replicative plasmids (Table S2) were grown at 37°C (TOP10 and NEB10 beta) or 30°C (CM404), on LB medium containing selective antibiotics: ampicillin (Amp) 100 µg.mL⁻¹, kanamycin (Km) 50 µg.mL⁻¹, streptomycin (Sm) 25 µg.mL⁻¹ or spectinomycin (Sp) 75 µg.mL⁻¹.

S.6803 and S.7002 were grown under continuous white light (2500 lux; 31.25 µE.m⁻².s⁻¹) and agitation (140 rpm, Infors rotary shaker) at 30°C, in liquid mineral medium: MM, *i.e.* BG11 [45] enriched with 3.78 mM Na₂CO₃ for S.6803 [46], or A+ supplemented with B12 vitamin (4 µg.L⁻¹) for S.7002 [47]. The terpene-producing strains were grown in the presence of the selective antibiotics: Km 50 µg.mL⁻¹ for both S.6803 and S.7002, Sm 5 µg.mL⁻¹ and Sp 5 µg.mL⁻¹ for S.6803, and Sm 50 µg.mL⁻¹ and Sp 50 µg.mL⁻¹ for S.7002. Growth was monitored by regular measurements of optical density at 750 nm (OD₇₅₀) with a spectrophotometer (Jenway 6700).

3.2. Genetic Manipulations and Gene Transfer Techniques

The studied cyanobacterial genes were amplified by polymerase chain reaction (PCR) from cyanobacterial DNA with specific oligonucleotide primers (Table S1) using Hot start Phusion polymerase (ThermoFisher Science). PCR products were digested with appropriate restriction enzymes and cloned either in the RSF1010-derived autonomously replicating pC vector [18] for high-level gene expression, or a commercial *E. coli* plasmid for gene deletion. All DNA constructions were verified by PCR and DNA sequencing (Mix2Seq Kit, Eurofins Genomics) using appropriate oligonucleotide primers (Table S1). The pC-derived plasmids (Table S2) were introduced in S.6803 and S.7002 by conjugation [8,10,48], using a 72h co-incubation of *E. coli* and cyanobacterial cells. The DNA deletion cassettes were introduced in S.6803 by transformation [49]. Cells were then plated on MM containing Km 50 µg.mL⁻¹ or both Sp 5 µg.mL⁻¹ and Sm 5 µg.mL⁻¹ and solidified with 1% Bacto Agar (Difco).

3.3. Terpenes Collection, and Quantification by Gas Chromatography–Mass Spectrometry

S.6803 and S.7002 engineered strains were grown photoautotrophically in the presence of selective antibiotics, in 250 mL erlenmeyers containing 50 mL cell suspensions covered with 20% (vol/vol) dodecane overlay (analytical grade, Sigma-Aldrich) to trap terpenes. At time intervals, 300 µL of these dodecane overlays were collected, and 1 µL of these samples were injected into a GC-MS apparatus (Trace1300 (GC) + ISQ LT (MS), ThermoScientific) using split modes of 10:1 (limonene) or 5:1 (farnesene). Terpenes were quantified as we previously described [7,8,10].

4. Conclusions

In this study, we have tested various genetic strategies to attempt at enhancing farnesene production in S.6803 and limonene production in S.7002. We report that the overexpression of the genes encoding the CO₂-fixing enzymes RubisCO and phosphoribulokinase increase the production of terpene in S.6803, not in S.7002. Furthermore, the overexpression of the *crtE* genes encoding the geranylgeranyl pyrophosphate synthase (synthesis of terpene precursors) from S.6803 or S.7002 enhance farnesene production in S.6803. In contrast, the overexpression of the *crtE* gene from S.6803,

but not S.7002, increase farnesene production in S.7002. Collectively these results emphasize the physiological difference between the two model cyanobacteria S.6803 and S.7002, which are often used for biotechnological projects. These differences remind us that because a model cyanobacterium is essentially a model of itself, it is important to study several cyanobacteria in parallel (in the same laboratory) to better understand their common and specific properties, and exploit this knowledge for biotechnological purposes. We also showed that, the deletion of the *crtR* and *cruF* genes (carotenoid synthesis) and *phaAB* genes (carbon storage) did not increase the production of farnesene in S.6803. Finally, we have tested a containment strategy of genetically modified (GMO). We report that the deletion of the *ccmK3K4* genes (carboxysome for CO₂ fixation) did not impact the production of limonene but decreased the production of farnesene in S.6803. It is clear from these results and other data in the literature that we need to better understand the metabolism of cyanobacteria to engineer strains for an economically-viable photoproduction of terpenes.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Physical map of the pC-derived plasmids expressing the studied *rbc* operons. Figure S2: Schematic representation of the farnesene-producing *Synechocystis* PCC 6803 strains overexpressing CO₂-fixation genes. Figure S3: Schematic representation of the limonene-producing *Synechococcus* PCC 7002 strains overexpressing CO₂-fixation genes. Figure S4: Schematic representation of the *Synechocystis* PCC 6803 strains producing farnesene or limonene in the presence, or not, of an extra *crtE* gene from *Synechocystis* PCC 6803 or *Synechococcus* PCC 7002. Figure S5: PCR verification of the pCFS-*crtE* and the pCLS-*crtE* replicative plasmids in *Synechocystis* PCC 6803. Figure S6: Schematic representation of the *Synechococcus* PCC 7002 strains producing farnesene in the presence, or not, of an extra *crtE* gene from *Synechococcus* PCC 7002 or *Synechocystis* PCC 6803. Figure S7: PCR verification of the pCFS-*CrtE* and pCLS-*CrtE* replicative plasmids in *Synechococcus* PCC 7002. Figure S8: Construction and analysis of the *DphaAB::KmR* mutant and their derivative reporter strains propagating the pCFS or pCLS plasmids. Figure S9: Schematic representation of the *Synechocystis* PCC 6803 producing farnesene or limonene in presence or absence of the *phaA-phaB* genes. Figure S10: Construction and analysis of the *DcrtR::KmR* mutant and its derivative reporter strain propagating the pCFS plasmid. Figure S11: Schematic representation of the *Synechocystis* PCC 6803 producing farnesene in presence or absence of the *crtR* or *cruF* genes. Figure S12: Construction and analysis of the *DcruF::KmR* mutant and its derivative reporter strain propagating the pCFS plasmid. Figure S13: Construction and analysis of the *DccmK3K4::KmR* double mutant. Figure S14: Schematic representation of the *Synechocystis* PCC 6803 producing farnesene or limonene in presence or absence of the *ccmK3-ccmK4* genes. Table S1: Characteristics of the oligonucleotide primers used in this study. Table S2: Characteristics of the bacterial strains and plasmids used in this study.

Author Contributions: Conceptualization, F.C. and C.C.-C.; methodology, F.C. and C.C.-C.; validation, M.V., C.C., V.-B.G., M.C., S.F., and L.F.G.-A.; formal analysis, M.V., C.C., V.-B.G., M.C., and S.F.; investigation, C.C., V.-B.G., M.V., M.C., S.F., C.C.-C. and F.C.; resources, F.C., C.C.-C. and L.F.G.-A.; writing—original draft preparation, F.C. and C.C.-C.; writing—review and editing, F.C., C.C.-C., M.V., C.C., V.-B.G. and L.F.G.-A.; supervision, F.C. and C.C.-C.; project administration, F.C. and C.C.-C.; funding acquisition, F.C., C.C.-C. and L.F.G.-A. All authors have read and agreed to the published version of the manuscript.

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